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PRINCIPAL INVESTIGATOR: Debabrata Mukhopadhyay, Ph.D.

CONTRACTING ORGANIZATION: Beth Israel Deaconess Medical Center
Boston, Massachusetts 02215

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6. AUTHOR(S)
Debabrata Mukhopadhyay, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Beth Israel Deaconess Medical Center
Boston, Massachusetts 02215

E-Mail: dmukhopa@caregroup.harvard.edu

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13. ABSTRACT *(Maximum 200 Words)*

Mammary tumors growth, like most of the other solid tumors, beyond minimal size requires the generation of new blood vesseles and other stromal elements. It is generally agreed that vascular permeability factor / vascular endothelial grwoth factor (VPF/VEGF) is thought to be largely responsible for the hyperpermeability of tumor blood vessels. Information accumulating from different laboratories including ours have indicated that tumor angiogenesis and stroma formation develop sequentially as increased microvascular permeability triggered by VPF/VEGF secreted mainly by the tumor. This seems to be the key initiation step of angiogenesis. We, therefore, postulate that by blocking the vascular permeability, the growth of solid tumors as well as its metastasis can be prevented. Utilizing Cre/lox system, we have palnned to generate mutant forms of VPF/VEGF in breast cancer cell lines (particularly MDA-MB435 cell line) which express either the potent vascular permeabilizing activity of native VPF/VEGF but is unable to stimulate endothelial cell division or lack of vascular permeabilizing acitvity but contains EC-mitogenic activity. Initial experiments are in progress by making those VEGF mutants into adneovirus vector and test whether the in vitro results can be reproducible in animal system. Meanwhile, in another project, we have defined the central role of p53 on regulation of VEGF in breast cancer.

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Annual Report:

Introduction

Angiogenesis measured in primary breast cancer is a major prognostic factor in both node-negative and node positive patients. Blood serum levels of VPF/VEGF in women with high-risk breast cancer are higher than in normal controls. Expression of VPF/VEGF mRNA detected by reverse transcriptase-polymerase chain reaction in breast tumors correlates with tumor-related characteristics of angiogenesis and metastatic potential. Interestingly, human breast cancer shows significantly higher vascularization than normal breast tissue, and there is a high degree of variability in the vascularization of individual tumors. The grade of vascularization is also correlated to the presence of metastatic deposits in lymph nodes, and the number of microvessels is associated with the number of positive nodes. Therefore, understanding of initiation of angiogensis and its overall impact on breast tumor grwoth and metastasis will generate the information which in turn will be helpful to develop new therapies for the treatment of breast cancer.

Although there have been several efforts made to elucidate how tumor cells promote angiogenic response, only handful of reports have described the molecular mechanisms of this response with respect to tumor cells. In this project, we will determine whether by inhibiting the vascular permeability or leakiness of tumor blood vessels only can inhibit breast tumor growth and metastasis.

Body

Previously, we found (Dr. R. deWaal is our collaborator) that substitution of cysteine 4 of the VPF/VEGF cysteine knot to serine (C4S) lose the EC-mitogenic activity but retains its vascular permeabilizing activity. On the other hand, "loop I swap" mutant where loop I of native VPF/VEGF has been substituted with the corresponding loop from PDGF retain its EC-mitogenic activity, but lose its capacity to increase vascular permeabilizing activity. Initially, we would like to reconfirm that result in a adenovirus system. Therefore, we subcloned both of the VEGF mutants in a adenoviral vector. We are doing collaboration with Dr. Harold F. Dvorak for this project and the rest of the expreiments are in progress.

In the meanwhile, we have tested another important aspects of angioenesis in breast cancer, that is regulation of VPF/VEGF. Hypoxia appears to be an important stimulus for inducing VPF/VEGF mRNA expression in human breast cancers. Here, we have studied the roles of the tumor suppressor gene p53 and the proto-oncogene c-Src in regulating the transcription of VPF/VEGF in breast cancer cell lines MCF-7 and MDA-MB 435 under both normoxic and hypoxic conditions. p53 significantly inhibited the transcription of VPF/VEGF involving the transcription factor Sp1. Increased binding of Sp1 to the VPF/VEGF promoter has been observed, when the cells were exposed to hypoxia. It has been shown that p53 makes a complex with Sp1 and inhibits its binding to the VPF/VEGF promoter to prevent the transcriptional activation. Furthermore, c-Src kinase activity was found to be increased in hypoxic condition and, in the presence of antisense of Src, there was downregulation of the total mRNA level and also the promoter activity of VPF/VEGF. The present study indicates that p53

can also inhibit the hypoxic induction of Src kinase activity and thereby may prevent VPF/VEGF transcription. Taken together, our data suggest a central role of p53, through which it can inhibit VPF/VEGF expression by regulating the transcriptional activity of Sp1 and also by downregulating the Src kinase activity, under both normoxic and hypoxic conditions.

Key Research Accomplishments

Our results implicate a central role of p53, a well known tumor suppressor gene, by which it can inhibit VPF/VEGF expression by regulating the transcriptional activity of Sp1 and also by inhibiting the Src kinase activity, under both normoxic and hypoxic conditions.

Reportable Outcomes

Recently we have published a paper in Cancer Research that was partly supported by this award (Copy of the reprint attached).

Soumitro Pal, Kaustubh Datta and Debabrata Mukhopadhyay, Central role of p53 on regulation of vascular permeability factor /vascular endothelial growth factor (VPF/VEGF) expression in mammary carcinoma. Cancer Research, 2001; **61**:6952-57.

(The first two authors have equal contributions)

Conclusions

The work are in progress.

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Mukhopadhyay D, Nagy JA, Manseau EJ, Dvorak HF.Vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) mediated signaling in mouse mesentery vascular endothelium. Cancer Res. 1998; 58:1278-1284.

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[CANCER RESEARCH 61: 6952–6957, September 15, 2001]

Central Role of p53 on Regulation of Vascular Permeability Factor/ Vascular Endothelial Growth Factor (VPF/VEGF) Expression in Mammary Carcinoma[1]

Soumitro Pal,[2] Kaustubh Datta,[2] and Debabrata Mukhopadhyay[3]

Departments of Pathology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts 02215

ABSTRACT

The process of angiogenic switching is one of the most important factors in the growth and development of breast tumors. Vascular permeability factor/ vascular endothelial growth factor (VPF/VEGF) is considered to be the most important directly acting angiogenic protein that has been shown to be up-regulated in breast cancer cells. Hypoxia seems to be an important stimulus for inducing VPF/VEGF mRNA expression in human mammary tumors. Here, we have studied the roles of the tumor suppressor gene *p53* and the proto-oncogene *c-Src* in regulating the transcription of VPF/VEGF in breast cancer cell lines MCF-7 and MDA-MB 435 under both normoxic and hypoxic conditions. p53 significantly inhibited the transcription of VPF/ VEGF involving the transcription factor Sp1. Increased binding of Sp1 to the VPF/VEGF promoter has been observed when the cells were exposed to hypoxia. It has been shown that p53 makes a complex with Sp1 and inhibits its binding to the VPF/VEGF promoter to prevent the transcriptional activation. Furthermore, c-Src kinase activity was found to be increased in the hypoxic condition, and in the presence of antisense of Src, there was down-regulation of the total mRNA level and also the promoter activity of VPF/ VEGF. The present study indicates that p53 can also inhibit the hypoxic induction of Src kinase activity and thereby may prevent VPF/VEGF transcription. Taken together, our data suggest a central role of p53, through which it can inhibit VPF/VEGF expression by regulating the transcriptional activity of Sp1 and also by down-regulating the Src kinase activity, under both normoxic and hypoxic conditions.

INTRODUCTION

The importance of tumor angiogenesis has been widely accepted for its role in the growth and development of solid tumors (1, 2). In most of the tumors, angiogenesis is mediated by several angiogenesis-promoting growth factors, such as VPF/VEGF,[4] platelet derived growth factor, transforming growth factor-α, and basic fibroblast growth factor (3–7). Importantly, breast tumors are not an exception (8, 9). VPF/VEGF is a secreted protein that plays a critical role in tumor-associated microvascular hyperpermeability and angiogenesis (3, 6). Some recent studies have demonstrated the importance of microvessel density for malignant progression in breast cancer, underscoring the importance of angiogenesis in this type of tumor (7, 10, 11). It has been shown that, in response to hypoxic conditions, human mammary fibroblasts dramatically up-regulate VPF/VEGF mRNA and increase VPF/VEGF protein levels in accordance with the degree of oxygen deprivation (12). Oncogenes and antioncogenes also play an important role in regulating VPF/VEGF expression and angiogenesis (13–16).

The genetic alterations responsible for oncogenesis and tumor progression may underline the ability of breast tumors to switch to an angiogenic phenotype (17). The *p53* tumor suppressor gene is one of the most frequently mutated genes in human cancers (18). The loss of p53 function, via somatic mutations or the expression of viral oncoproteins, contributes to the activation of the angiogenic switch during tumorigenesis (13, 15, 17, 19). We have shown that wt *p53* can significantly inhibit the VPF/VEGF transcriptional activation, although the exact mechanism of this inhibition was not explored (13). Human *p53* encodes a multifunctional transcription factor that mediates cellular responses to diverse stimuli, including DNA damage and hypoxia (20). In addition to being an integral component of the surveillance mechanisms that arrests cell cycle progression under adverse conditions, p53 is also involved in mediating hypoxia-induced apoptosis (21) and inducing inhibitors of angiogenesis such as thrombospondin-1 (22, 23). It has been reported that microvessel density, p53 expression, tumor size, and peritumoral lymphatic vessel invasion are relevant prognostic markers in node-negative breast carcinomas (24).

Amplification and overexpression of the *neu* (*c-erbB2*) proto-oncogene has been implicated in the pathogenesis of 20–30% of human breast cancers (25–27). Although the activation of Neu receptor tyrosine kinase appears to be a pivotal step during mammary tumorigenesis, the mechanism by which Neu signals cell proliferation is unclear. Molecules bearing a domain shared by the *c-Src* proto-oncogene (Src homology 2) are thought to be involved in signal transduction from activated receptor tyrosine kinases such as Neu (28). The *polyomavirus middle T* oncogene in the mammary epithelium develop multifocal mammary tumors that metastasize with high frequency (29). It has been reported that the potent transformational activity of polyomavirus middle T antigen is also attributed to its ability to associate with and to activate a number of c-Src family tyrosine kinases (c-Src, c-Yes, and Fyn; Refs. 29 and 30). Recent reports have indicated that human breast carcinomas contain functionally activated pp60c-Src (31, 32). In previous studies, we have shown that c-Src activation by hypoxia up-regulates VPF/VEGF expression, and constitutive v-Src increased VPF/VEGF mRNA (33). Thus, Src family kinases seem to play a significant role in the advancement of human breast tumors (31, 34). We have also reported that wt p53 and v-Src exert an opposing influence on *VPF/VEGF* gene expression (13).

In the present study, we examine in more detail the mechanistic role of *p53* and *c-Src* in regulating the transcriptional expression of VPF/ VEGF in breast cancer cell lines MCF-7 and MDA-MB 435 under both normoxic and hypoxic conditions. We demonstrate that p53 makes a complex with Sp1 and thereby inhibits Sp1-mediated VPF/ VEGF transcriptional activation. *p53* also plays a significant role in down-regulating hypoxia-induced Src kinase activity.

MATERIALS AND METHODS

Cell Culture. Human breast carcinoma cell lines, MCF-7 and MDA-MB 435 were maintained in DMEM with 10% fetal bovine serum (Hyclone Laboratories).

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[2] The first two authors have contributed equally to this work.

[3] To whom requests for reprints should addressed, at Departments of Pathology, Beth Israel Deaconess Medical Center and Harvard Medical School, 330 Brookline Avenue, RN 270H, Boston, MA 02215. Phone: (617) 667-7853; Fax: (617) 667-3591; E-mail: dmukhopa@caregroup.harvard.edu.

[4] The abbreviations used are: VPF/VEGF, vascular permeability factor/vascular endothelial growth factor; wt, wild type; EMSA, electrophoretic mobility shift analysis; HIF-1, hypoxia-inducible factor 1.

Plasmids. The *VPF/VEGF* reporter constructs used in transient transfection assays contain sequences derived from human VPF/VEGF promoter driving expression of firefly luciferase. The 0.35- and 0.07-kb deletion mutant constructs were made by PCR from the 2.6-kb promoter fragment and subcloned into pGL-2 basic vector (Promega), as described earlier (14). Human cytomegalovirus immediate-early promoter-driven wt and mutant p53 expression plasmids (generously provided by Prof. Arnold J. Levine, Princeton University, Princeton, NJ) were used in this study. wt *p53* contains wt *p53* cDNA, whereas p53-SCX3 is a single-nucleotide mutation that results in a valine to alanine alteration at codon 143 in *p53*-wt, and p53–4.2N3 is a mutant human *p53* cDNA derived from an epidermoid carcinoma cell line A431 (13). This *p53* gene encodes an arginine to histidine change at codon 273. Src sense and antisense expression vectors were generous gifts from G. E. Gallick (The University of Texas, M.D. Anderson Cancer Center, Houston, TX; Ref. 35).

Transfection Assays. Cells were plated at 2–3 $\times$ 10^5 cells/60-mm dish 1 day before transfection with VPF/VEGF promoter-luciferase construct and expression plasmids using calcium-phosphate precipitation (36). The expression was normalized with a control empty-expression vector. Cells were harvested for luciferase assay 40 h after transfection. In all cotransfection experiments, transfection efficiency was normalized by assaying β-galactosidase activity using the *β-galactosidase* gene under control of the cytomegalovirus immediate-early promoter as internal control.

Immunoprecipitations and Western Blot Analyses. Cells were washed twice with cold PBS, lysed with ice cold lysis buffer [50 mM Tris (pH 7.5), 1% NP40, 150 mM NaCl, 1 mM $NaVO_4$, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 0.5% aprotinin, and 2 mM pepstatin A], incubated for 10 min on ice, and centrifuged for 10 min at 4°C. Immunoprecipitations were carried out at antibody excess using 0.5 mg of total protein with rabbit polyclonal antibodies (1 μg) directed against p53 (Santa Cruz Biotechnology) and Sp1 (Santa Cruz Biotechnology). Immunocomplexes were captured with protein A agarose beads (Pharmacia). After three washes with cell lysis buffer, bead-bound proteins were separated by SDS-PAGE. Western blot analysis was carried out as described earlier (14).

Kinase Assay. Src kinase activity was assayed by measuring the incorporation of ^{32}P into a Src-specific peptide (33). The cellular extracts were immunoprecipitated with Src-specific antibody (Santa Cruz Biotechnology) and the immunoprecipitates were incubated in a 25 μl-reaction mixture consisting of 30 mM Tris-HCl (pH 7.5), 0.01% Triton X-100, 10 mM 2-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 10 mM $MgCl_2$, 0.4 mM Src-specific peptide (Upstate Biotechnology, Inc.), and 50 μM [γ-^{32}P]ATP for 30 min at 30°C. The reaction was stopped by the addition of ice-cold 25% trichloroacetic acid. Precipitates were collected on phosphocellulose filter paper. The filters were washed with 0.75% phosphoric acid and counted for ^{32}P using liquid scintillation spectroscopy.

Nuclear Extract Preparation and EMSAs. Nuclear extracts were prepared using MCF-7 cells following a standard protocol, with modifications (14, 16). Cells were washed in cold PBS, suspended in buffer A [10 mM HEPES (pH 7.8), 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 μg/ml aprotinin, 3 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride] and incubated for 15 min on ice. Cells were then lysed with 0.5% NP40 and the pellets were resuspended in buffer C [50 mM HEPES (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 3 mM DTT and 0.1 mM phenylmethylsulfonyl fluoride]. After incubation on a rotating rack for 25 min, samples were centrifuged at 14,000 RPM for 10 min. Clear supernatants containing the nuclear proteins were collected and stored at −70°C.

EMSAs were performed as described previously (14, 16). Briefly, EMSA binding reaction mixtures (25 μl) contained 20 mM HEPES (pH 8.4), 100 mM KCl, 20% glycerol, 0.1 mM EDTA, 0.2 mM $ZnSO_4$, 0.05% NP40, and 1 μg BSA. Extract protein and 200 ng of poly(dA-dT).poly(dA-dT) were added at room temperature 10 min before the addition of ~0.1 ng of radiolabeled oligonucleotide probe. After 20 min incubation at 4°C, samples were run on 7% acrylamide gel in 1× TAE (40 mM Tris-acetate and 1 mM EDTA) buffer.

The radiolabeled oligonucleotide used in EMSA studies was a 188-bp PCR-generated fragment (bp −195 to −7, relative to the transcription start site) of the VPF/VEGF promoter containing the four putative Sp1 binding sites (14, 16).

RNA Analysis by Northern Blot Hybridization. Total RNA, isolated by the single-step acid-phenol extraction method (37), was separated on a formaldehyde-agarose gel, transferred to a GeneScreen (DuPont) membrane by using 10× SSC, and probed with random-primer-labeled cDNAs in a solution containing 0.5 M sodium phosphate (pH 7.2), 7% SDS, 1% BSA, 1 mM EDTA, and sonicated herring sperm DNA (50 μg/ml) at 68°C. Blots were washed three times with a solution containing 40 mM sodium phosphate (pH 7.2), 0.5% SDS, 0.5% BSA, and 1 mM EDTA at 68°C and autoradiographed.

RESULTS

p53 Inhibits Sp1-mediated Transcription of VPF/VEGF in Breast Cancer Cells. It has been shown previously that overexpression of the tumor suppressor wt *p53* can inhibit transcription of many cellular and viral promoters (38). Interestingly, wt *p53* specifically repressed the activity of TATA promoters that do not contain a wt *p53* DNA binding sequence (39). The VPF/VEGF promoter does not possess a TATA box or wt *p53* DNA binding sequence, but it does contain five Sp1 binding sites (40). Although we have shown previously that wt *p53* can inhibit the VPF/VEGF promoter activity, the mechanism of this inhibition is still unclear (13). Here, we sought to determine whether wt *p53* is involved in Sp1-mediated transcriptional regulation of VPF/VEGF in human breast cancer cells. To this end, MCF-7 cells were cotransfected with a 2.6-kb VPF/VEGF promoter-luciferase construct and plasmid containing wt *p53* cDNA. VPF/VEGF reporter activity was decreased by 60% in comparison with the cells transfected with expression vector alone (Fig.1*A*). To define the region of the VPF/VEGF promoter that is responsive to wt *p53*, we used two different 5′ deletions of the 2.6-kb promoter-reporter vector and cotransfected these deletions with the plasmid expressing wt p53. It has been found that wt p53 decreased the reporter activity by 70% in the 0.35-kb segment of the *VPF/VEGF* promoter that contains multiple Sp1 binding sites, although there was no change of reporter activity in the 0.07-kb VPF/VEGF promoter having the deleted Sp1 binding site (Fig.1*A*). We also observed a similar result in MDA-MB 435 cells (data not shown). These observations reveal that in breast cancer cells, wt *p53* may inhibit VPF/VEGF transcription by interfering with Sp1-mediated promoter activation. Interestingly, when we used two different mutants of wt *p53*, they could not down-regulate the Sp1-mediated *VPF/VEGF* promoter activity in MCF-7 cells (Fig.1*B*).

p53 Associates with Sp1 and Prevents Its Binding to the VPF/VEGF Promoter. Because we have observed that wt p53 inhibits Sp1-mediated VPF/VEGF transcription, here we set out to determine whether, in breast cancer cells, wt p53 might form a complex with Sp1. To test this possibility, we transfected both MCF-7 and MDA-MB 435 cells with the expression vector containing wt p53. Lysates of transfected and nontransfected cells were immunoprecipitated with affinity-purified antibodies to wtp53, and then Western blotting with antibody directed against Sp1 was performed. We found a strong band corresponding to Sp1 in the cases of the transfected samples of both cell lines (Fig.1*C*).

To examine the consequences of the association between p53 and Sp1 on VPF/VEGF transcription, we performed EMSA by using a 188-bp VPF/VEGF promoter fragment containing all four Sp1 binding sites, with the nuclear extracts of MCF-7 cells transfected with or without wt p53. As shown in Fig.1*D*, wt p53 inhibited the binding of Sp1 to the VPF/VEGF promoter. This specific protein-DNA complex formation was competed away with 10-fold molar excess of Sp1 consensus oligonucleotide. From these results, it seems that wt p53 forms a complex with Sp1 and, thereby, may prevent the VPF/VEGF transcriptional activation by blocking Sp1 and promoter interaction.

p53 Can Also Down-Regulate Hypoxia-induced Activation of VPF/VEGF Transcription. Hypoxia appears to be an important stimulus in promoting the overexpression of VPF/VEGF. Previous studies

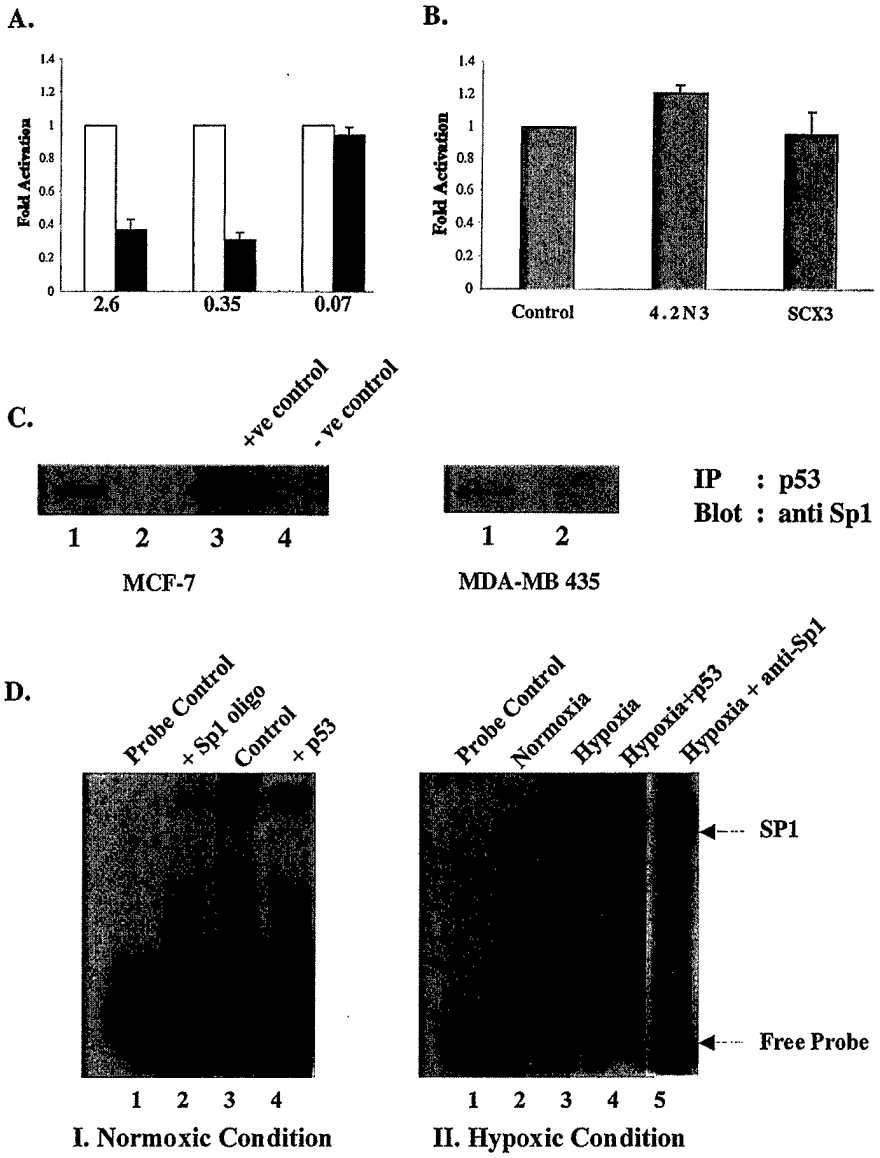

Fig. 1. Effect of *p53* on Sp1-mediated transcriptional activation of VPF/VEGF. *A*, MCF-7 cells were cotransfected with 2.6-, 0.35-, or 0.07-kb VPF/VEGF promoter-luciferase constructs (1.0 μg) and wt p53 (0.5 μg) expression vectors. Cells were harvested for luciferase assays 40 h after transfection, and fold activation was calculated as relative to the activity of same reporter construct cotransfected with an empty expression vector. □, empty expression vector; ■, wt *p53* expression. *B*, MCF-7 cells were cotransfected with 0.35-kb VPF/VEGF promoter-luciferase construct (1.0 μg) and mutant *p53* (p53-SCX3 and p53–4.2N3) expression vectors. After harvesting the cells, luciferase activity was measured as described above. *C*, extracts were prepared from MCF-7 and MDA-MB 435 cells and transfected with wt p53 expression vector. Both the transfected and untransfected cell lysates were immunoprecipitated with a polyclonal antibody directed against wt p53. All immunoprecipitates were then captured by protein A-Sepharose beads. After thorough washings, the Sepharose beads were boiled in SDS buffer and separated by SDS-PAGE. Western blottings (*Blot*) were performed by using Sp1 polyclonal antibody. *Lane 1*, transfected with wt p53 expression vector; *Lane 2*, transfected with empty expression vector; *Lanes 3* and *4*: control cell lysates, immunoprecipitated with polyclonal antibodies, directed against Sp1 and IGF-1 respectively. *D*, by using a 188-bp VPF/VEGF promoter fragment (having all of the four Sp1 binding sites) as the probe, EMSA was performed with partially purified nuclear extracts of MCF-7 cells, transfected with wt p53 (0.5 μg), and subjected to both normoxic (*I*) and hypoxic conditions (*II*). In hypoxic conditions, the cells were exposed to hypoxia overnight. Nuclear extracts were prepared from the transfected cells 40 h after transfection. *Lane 1*, probe control, without any nuclear extract. In *Lane 2* of the normoxic condition, the unradiolabeled Sp1 consensus oligonucleotide (*oligo*; 10-fold molar excess) was added to the binding reaction mixture of the control sample (without any transfection) to show that it can compete away the specific protein-DNA complexes. In *Lane 5* of the hypoxic condition, Sp1 polyclonal antibody (2 μg) was added to the reaction mixture of the hypoxic sample to show the supershift of the Sp1 band.

have indicated that p53 can down-regulate hypoxia-mediated VPF/VEGF transcription through degradation of HIF-1α (17). Here, we attempted to explore whether wt p53 can also inhibit the hypoxic induction of VPF/VEGF transcriptional activation involving Sp1. To this end, MCF-7 cells were transfected with 2.6-kb promoter-reporter construct (containing both HIF-1α and Sp1 binding sites) and wt p53 and then subjected to hypoxic condition. As shown in Fig. 2, wt p53 clearly down-regulated the hypoxia-mediated activation of VPF/VEGF transcription as compared with the normoxic control. We found a similar result in MDA-MB 435 cells (data not shown). Additionally, we also observed through EMSA that, under hypoxic condition, there was increased binding of Sp1 to the VPF/VEGF promoter and, in the presence of wt p53, this binding was significantly inhibited (Fig. 1*D*). We have also confirmed the binding of Sp1 through antibody supershift study. Thus, from all these observations it is evident that, as under normoxic conditions, wt p53 plays a significant role in regulating Sp1-mediated VPF/VEGF transcription under hypoxic conditions. Interestingly, we did not get any significant hypoxic induction of the 0.35-kb VPF/VEGF promoter sequence, which, in spite of having Sp1 binding sites, does not contain any binding sequence for HIF-1α (data not shown). This observation indicates that a cooperative function of both Sp1 and HIF-1α may be needed for VPF/VEGF transcriptional activation under hypoxic conditions.

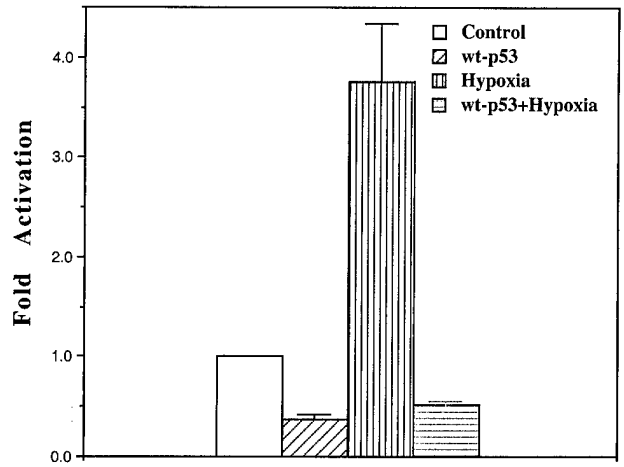

Fig. 2. Effect of p53 on hypoxia-induced activation of VPF/VEGF transcription. MCF-7 cells were cotransfected with 2.6-kb VPF/VEGF promoter-luciferase construct (1.0 μg) and wt p53 expression vector (0.5 μg). One set of cells was subjected to the hypoxic condition for overnight. Cells were harvested for luciferase assays 40 h after transfection, and fold activation was calculated as relative to the activity of the same reporter construct cotransfected with an empty expression vector.

Activation of Src Kinase Activity in Breast Cancer Cells Under Hypoxia. Hypoxia is a potent inducer of tyrosine kinase cascade, and the activation of tyrosine kinases has been found to be critical in signaling triggered by growth factors (33). Here, we measured the Src kinase activity in MDA-MB 435 cells under hypoxic conditions. The breast cancer cells were exposed to hypoxia for 15, 30, and 60 min, respectively. The cell extracts were used to perform Src kinase assays using an Src-specific peptide as a substrate. As shown in Fig. 3*A*, the substrate was strongly phosphorylated when the cells were exposed to hypoxic condition for 30 min, although the Src protein levels were the same in all of the conditions (Fig. 3*B*). This result suggests that Src, activated by hypoxia, is a key signaling molecule in human breast cancer cells.

c-Src Promotes Increased VPF/VEGF mRNA Levels in Breast Cancer Cells Subjected to Hypoxia. To analyze the role of *c-Src* on hypoxia-induced *VPF/VEGF* gene expression in human breast carcinoma, MCF-7 cells were transfected with Src antisense and sense cDNA containing expression vectors and afterward subjected to hypoxia. Interestingly, in Northern blot analysis, it has been observed that the hypoxic induction of VPF/VEGF mRNA expression was effectively blocked in Src antisense-transfected MCF-7 cells as compared with the Src sense-transfected ones (Fig. 4). We also observed a similar type of inhibition of hypoxia-induced VPF/VEGF expression in MDA-MB 435 cells using Src antisense cDNA (data not shown). This result clearly suggests the importance of c-Src in increasing the VPF/VEGF mRNA level in human breast cancer cells under hypoxic conditions.

c-Src Induces Hypoxia-mediated Transcriptional Activation of VPF/VEGF. As the activation of c-Src has been found to be an important factor in the regulation of hypoxia-induced up-regulation of VPF/VEGF, we set out to determine whether the antisense of Src could block the hypoxia-induced VPF/VEGF transcriptional activation. Indeed, Fig. 5 shows that in MCF-7 cells cotransfected with 2.6-kb promoter-luciferase construct and Src antisense cDNA, the hypoxia-induced VPF/VEGF transcriptional activation was significantly reduced in comparison to the cells transfected with Src sense cDNA. This result indicates that in breast cancer cells, *c-Src* activation is critical for hypoxia-mediated up-regulation of VPF/VEGF transcription.

p53 Can Down-Regulate Hypoxia-induced Src Kinase Activity in Breast Cancer. Inasmuch as *p53* plays an important role in regulating tumor angiogenesis and because *c-Src* activity seems to be crucial for transcriptional expression of VPF/VEGF in breast cancer cells under hypoxia, we tried to examine whether *p53* could inhibit Src kinase activity in the MDA-MB 435 cell line. Src kinase activity was measured in the *p53*-transfected cells, subjected to both normoxic and hypoxic conditions. Interestingly, *p53* significantly decreased the normoxic as well as hypoxic induction of Src kinase activity (Fig. 6).

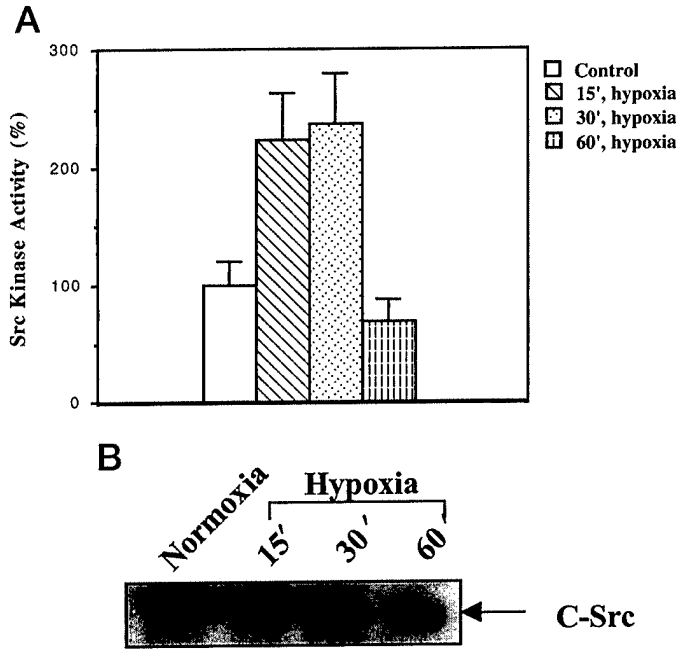

Fig. 3. Stimulation of Src kinase activity in breast cancer cells, subjected to hypoxia. *A*, MDA-MB 435 cells were subjected to either normoxic (*Control*) or hypoxic conditions for different time intervals. Kinase assay for *c-Src* was performed using specific substrate peptide for Src kinase. The percentage of activation of each experiment was determined in comparison with [γ-^{32}P]ATP incorporation under a normoxic condition, which was considered as 100% (actual reading was 2584 cpm). Results were the average of three independent experiments. *B*, expression of *c-Src* in MDA-MB 435 cells, subjected to the above conditions has been shown.

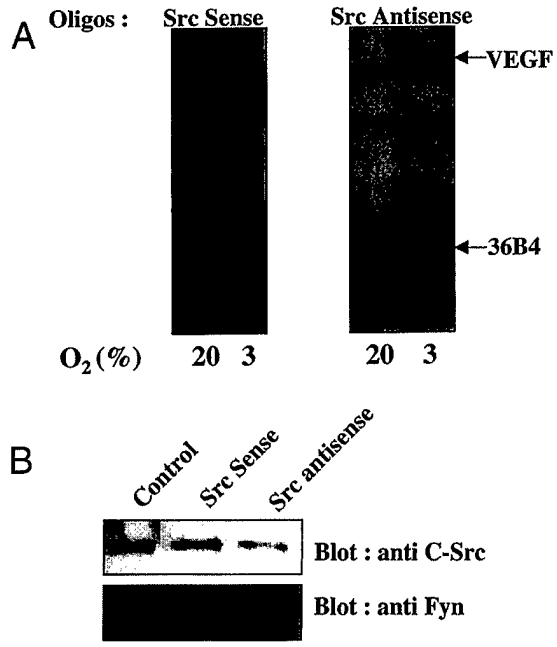

Fig. 4. Effect of c-Src on VPF/VEGF mRNA expression in breast cancer cells under hypoxia. *A*, total RNA (5 μg) was extracted from MCF-7 cells and transfected with Src antisense and sense cDNA containing expression vectors. The cells were exposed to both normoxic and hypoxic conditions. Northern blot analysis was performed by using ^{32}P-labeled *VPF/VEGF* cDNA. Fold expression was calculated by densitometry using 36B4 ribosome-associated mRNA expression as a normalized control. *B*, expression of c-Src and Fyn has been shown in Src sense- and antisense-transfected samples of MCF-7 cells.

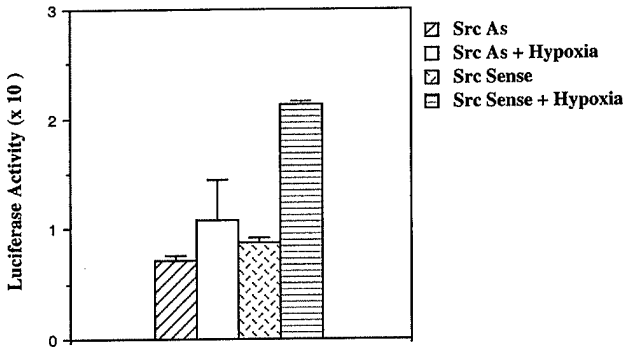

Fig. 5. Effect of Src antisense and sense cDNA on VPF/VEGF promoter activity in breast cancer cells under hypoxia. MCF-7 cells were cotransfected with 2.6-kb VPF/VEGF promoter-luciferase construct (1.0 μg) and Src-antisense (3.0 μg) or sense (3.0 μg) cDNA using the calcium phosphate precipitation method. After 24 h of transfection, one set of each type of transfected cell was subjected to hypoxic conditions for 6 h. Afterward, all of the cells were harvested, and luciferase assays were performed as described previously.

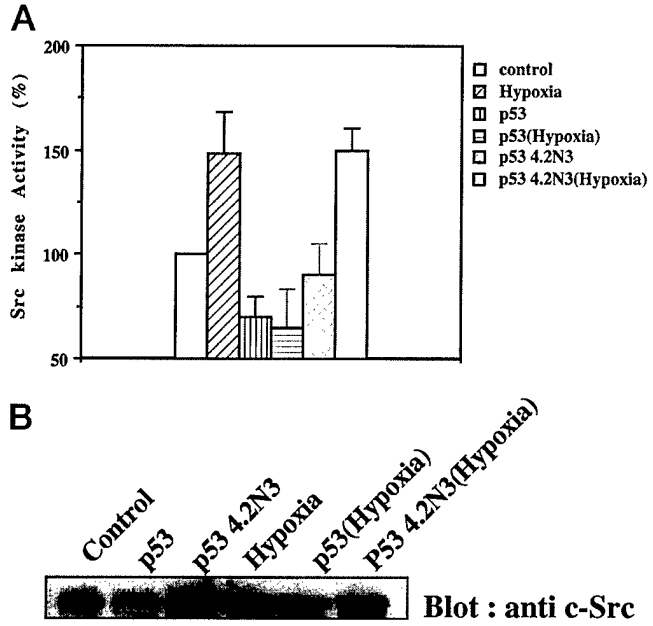

Fig. 6. Effect of p53 on Src kinase activity of breast cancer cells, subjected to both normoxic and hypoxic conditions. *A*, MDA-MB 435 cells were transfected with wt- *p53* or mutant *p53* (4.2N3) expression vector (0.5 μg). After 24 h of transfection, one set of cells from both transfected and untransfected groups were subjected to hypoxic conditions overnight. Afterward, a kinase assay of c-Src was performed using specific substrate peptide for Src kinase. The percentage of activation of each experiment was determined in comparison with [γ-^{32}P]ATP incorporation under normoxic conditions, which was considered as 100%. Results were the average of three independent experiments. *B*, expression of *c-Src* has been shown in all of the transfected samples of MDA-MB 435 cells.

The mutant *p53* could not decrease either normoxic or hypoxic induction of Src kinase activity (Fig. 6). This result clearly suggests that *p53* may play an important role in regulating tumor angiogenesis by inhibiting Src kinase activity under both normoxic and hypoxic conditions.

DISCUSSION

The genetic alterations involved in tumorigenesis are responsible for the phenotypic characteristics of cancer cells (17, 18, 41). The process of tumor angiogenesis is tightly controlled by the balance of positive and negative regulatory pathways (13, 42). The molecular mechanism of the angiogenic switch is a fundamental determinant of breast tumor growth and progression (7, 10, 11). Although several factors have been described as the inducer of tumor angiogenesis, VPF/VEGF is considered as the most important directly acting angiogenic cytokine (3, 6). Recent clinical studies have demonstrated quantification of intratumoral VPF/VEGF levels may be useful to assess the angiogenic phenotype of breast tumors (7, 10, 11). It plays an important role in vasculogenesis and in both pathological and physiological angiogenesis. Although constitutively expressed by many tumor cells, transformed cell lines, and some normal cells, VPF/VEGF expression is substantially up-regulated by hypoxia, cytokines, hormones, and certain oncogenes including activated forms of *Ras* and *Src* (15, 16, 33). Very little is known about the regulation of VPF/VEGF expression in breast cancer cells. In the present study, we have demonstrated that the antioncogene *p53* plays an important role in regulating breast tumor angiogenesis through the inhibition of VPF/VEGF transcriptional activation.

The tumor suppressor gene *p53* has been found to be mutated in most human cancers (17, 18, 43). It inhibits the proliferation of normal as well as transformed cells by interacting with viral and cellular oncoproteins (38). Overexpression of wt *p53* can inhibit transcription of many cellular and viral promoters. We have previously shown that wt *p53* can inhibit the transcription of VPF/VEGF (13). The present study elucidates a novel mechanism by which *p53* inhibits the transcription of VPF/VEGF in an Sp1-dependent manner in the two breast cancer cell lines MCF-7 and MDA-MB 435 (Fig. 1, *A* and *B*). *p53* was able to inhibit the VPF/VEGF promoter activity only if its Sp1 binding sites were intact (Fig. 1*A*). There was no change in the activity of the promoter with the deleted Sp1 binding sites (Fig. 1*A*). It has been shown that *p53* interacts with the transcription factor Sp1 and thereby prevents its binding to the VPF/VEGF promoter for its transcriptional activation (Fig. 1*C* and *D*). Thus, Sp1 seems to be the key target molecule for *p53* in regulating VPF/VEGF transcription.

Breast tumors, being solid in nature, contain regions of vascular deficiency or defective microcirculation and thereby are deprived of oxygen, glucose, and other nutrients (10, 11). Hypoxic regions of these solid breast tumors produce the powerful and directly acting angiogenic protein VPF/VEGF (8, 9). It is known that HIF-1, a heterodimeric transcription factor, is the key molecule that generally regulates cellular energy metabolism and angiogenesis in response to oxygen deprivation (17, 44). Some earlier studies have shown that wt *p53* does not repress hypoxia-induced transcription of VPF/VEGF (45). Interestingly, some later studies have demonstrated that *p53* promotes the proteasomal degradation of the α subunit of HIF-1 and thereby inhibits angiogenesis by down-regulating VPF/VEGF transcription (17, 46). In this study, we report that, like HIF-1α, Sp1 also plays a significant role in promoting VPF/VEGF transcription in breast cancer cells under hypoxic conditions (Fig. 1*D*). We have shown that, in this condition, there is increased binding of Sp1 to the VPF/EGF promoter (Fig. 1*D*). We have also demonstrated that, as under normoxic conditions, *p53* can also down-regulate the hypoxic induction of VPF/VEGF transcriptional activation in breast cancer cells by preventing the binding of Sp1 to its promoter (Figs. 1*D* and 2). The exact role of HIF-1α in this situation needs to be explored.

It has been reported that higher activity of the proto-oncogene *c-Src* is necessary for the increased expression of VPF/VEGF in different tumor cell lines, including glioma (47), colon carcinoma (35), ovarian carcinoma (48), lung carcimoma (49), and also in primary culture like mouse brown adipocytes (50) or in retinal vascular disorder (51). This phenomenon was observed not only in hypoxia but also in growth factor-regulated or X-ray- or C-ion-induced VPF/VEGF expression (49, 50, 52). In the present paper, we report increased c-Src kinase activity in MDA-MB 435 cells under hypoxia (Fig. 3). In breast cancer cell lines, c-Src is an important modulator of VPF/VEGF transcription under hypoxia because, in the presence of the antisense of Src, there was a down-regulation of the total mRNA level and also of the promoter activity (Figs. 4 and 5). Previously, we have reported that *v-Src* in presence of wt *p53* was unable to activate the transcription of the VPF/VEGF promoter (13). The present study suggests that wt *p53*, apart from its inhibitory role on the transcription factor Sp1, may also regulate *c-Src* activity and thereby down-regulate VPF/VEGF transcription, either directly or indirectly (Fig. 6). To understand the function of *p53* and *c-Src* in breast cancer cells, we transiently overexpressed wt *p53* as well as its dominant-negative mutant in MCF-7 and MDA-MB 435 cell lines, and we determined their Src kinase activity in both normoxic and hypoxic conditions (Fig. 6). The observation of down-regulation of c-Src kinase activity, only through wt *p53*, has led us to conclude that *p53* has a more direct effect on the inhibition of VPF/VEGF transcription by *c-Src* ,and that this inhibitory function is probably different from its effect on Sp1.

In conclusion, here we define a central inhibitory role of *p53* on the transcriptional regulation of the angiogenic growth factor VPF/VEGF in two different breast cancer cell lines. *p53* can directly regulate the transcriptional activity of Sp1 by inhibiting its association to the promoter

region of the *VPF/VEGF* gene, under both normoxic and hypoxic conditions. *p53* also regulates c-Src kinase activity and thereby inhibits c-Src-induced VPF/VEGF expression under the above conditions.

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